

## Research Article

# Cloning of the IPNV VP2 gene into pNZ8150/*Lactococcus lactis* expression system: A preliminary phase for the development of IPN vaccines for fish

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### Abstract

Infectious pancreatic necrosis virus (IPNV) is one of the major worldwide leading causes of losses among different fish species, especially in salmonidae. In order to develop a vaccine (First stage), the VP2 gene from an Iranian IPNV isolate was amplified using polymerase chain reaction (PCR), inserted between *SpeI* and *ScaI* restriction sites of pNZ8150 expression vector under the control of NICE promoter, and transformed into electrocompetent *Lactococcus lactis* cells using electroporation method regarding production of a recombinant live vector vaccine in fish. The transformed *L. lactis* cells containing the highest copy of the recombinant plasmid, pNZVP2, were selected using high antibiotic concentration (100 µg/mL of chloramphenicol). These cells are expected to express high level of VP2 protein and consequently will be the best candidates for vaccine formulation. Restriction enzyme digestion using *SpeI*, colony PCR using gene specific primers and boiling method, and DNA sequencing confirmed construction of the recombinant expression vector, pNZVP2. The present research will pave the way for expression and oral delivery of not only IPNV antigens, but also, any other infectious agents via pNZ8150/*L. lactis* expression system, potentially as a novel platform for the development of oral delivery vaccines in fish.

**Keywords:** Fish, Recombinant vaccine, Cloning, IPNV, VP2 gene, *Lactococcus lactis*

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## Introduction

Infectious pancreatic necrosis (IPN) is a highly contagious acute enteritis caused by the infectious pancreatic necrosis virus (IPNV) (Ahmadivand *et al.*, 2020), which is one of the most widespread viruses in aquaculture and in nature. This virus has been found to infect more than 63 species of marine aquatic animals, including fish, crustaceans, and mollusks (Ahmadivand *et al.*, 2018), causing widespread mortality in fry and juveniles of farmed fish (Hua *et al.*, 2021). Depending on the environmental condition, host species, virus species, and the propagation of IPNV, the disease may result in the deaths of 5 to 100% of the fish population (Ahmadivand *et al.*, 2018).

The IPNV belongs to the genus Aquabirnavirus and the family Birnaviridae and is a non-enveloped virus harboring a bi-segmented dsRNA genome, including "A" as the larger segment and "B" as the shorter (5 Kbp) segment (Ahmadivand *et al.*, 2020). The segment A, with approximately 3.1 kb in length has two open reading frames (ORF). The short ORF produces a 12-17 KDa nonstructural polypeptide, VP5, (Ballesteros *et al.*, 2015; Dadar *et al.*, 2014). The long open-reading frame (ORF) encodes a virion-associated RNA-dependent RNA polymerase (VPI) (Ahmadivand *et al.*, 2020). The long ORF encodes a 106 KDa precursor polyprotein (NH<sub>2</sub>-VP2-VP4-VP3-COOH) that is co-translationally cleaved by a viral protease, VP4, into pre-VP2 (62 KDa) and VP3 (31 KDa).

Following capsid assembly, pre-VP2 is further processed by host cell proteases generating a mature outer capsid protein VP2 (54 KDa) (Petit *et al.*, 2000; Cuesta *et al.*, 2010), which builds roughly 60% of virion (Dobos, 1995; Dadar *et al.*, 2014). Among the five proteins of IPNV, VP2 capsid protein is a type-specific antigen which contains most of the neutralizing epitopes of IPNV inducing neutralizing antibodies in the host (Fridholm *et al.*, 2007; Frost *et al.*, 1998; Tarrab *et al.*, 1995).

IPNV has been detected in Europe (Roberts and Pearson, 2005) especially in Norway and Shetland Isles of Scotland (Smail *et al.*, 2006; Stagg and Skjelstad, 2003; Smail *et al.*, 1992, Bowden *et al.*, 2002), North America (Molloy *et al.*, 2013, Roberts and Pearson, 2005), South America (Manríquez *et al.*, 2017; Tapia *et al.*, 2015), Australia (Crane *et al.*, 2000; Davies *et al.*, 2010), and in a very recently widespread outbreak occurred in farms of cultured rainbow trout fry in China, the virus resulted in high mortality (nearly 100%) in 2016 (Zhu *et al.*, 2017). In Iran, the first report on the detection of IPNV by RT-PCR was published in 2007 (Akhlaghi and Hosseini, 2007). After that, a number of studies reported outbreak of IPNV, with a considerable mortality in different provinces of Iran (Ahmadi *et al.*, 2013; Akhlaghi and Hosseini, 2007; Oryan *et al.*, 2012, Raissy *et al.*, 2010).

Hence, this disease is limiting the freshwater aquaculture industry, due to its worldwide dispersal and the important economic losses (Ballesteros

*et al.*, 2015). As there is no definite and effective treatment to eradicate IPNV, early diagnosis and prevention play crucial roles in fighting against the disease. In this regard, vaccination is an important strategy to control the disease and prevent deaths due to IPN. Various forms of IPNV vaccines (e.g., inactive, live, recombinant, DNA, etc.) have been used in developed countries (Tamer *et al.*, 2021). However, there are reports that the inactivated and subunit vaccines commercially available for the disease can promote an immune response in vaccinated fish, with some fish becoming carriers after vaccination (Ahmadivand *et al.*, 2018). In this regard, a very recently oral DNA vaccine based on CS-TPP nanoparticles and alginate microparticles induced great protection against IPNV and could patented by Iranian authorization (Soltani *et al.*, 2016; Ahmadivand *et al.*, 2017). Using DNA vaccines is an effective strategy to prevent viral diseases, such as IPN, in fish (Naderi-Samani *et al.*, 2020). DNA vaccines contain antigen-encoding DNA plasmids and are administered intramuscularly. Nevertheless, there have been conflicting results after the use of the majority of the plasmid-based commercial IPN vaccines available on the market upon intramuscular administration (Ahmadivand *et al.*, 2018).

However, as IPNV is very contagious and destructive to juvenile fish and is responsible for high mortality nearly 70% in hatchery stocks (Cuesta *et al.*, 2010; Wolf *et al.*, 1960;

Biering *et al.*, 2005; Roberts and Pearson, 2005), these vaccine formulations cannot be utilized for fingerling stages and oral administration is more preferred for early vaccination (Min *et al.*, 2012). In this regard, oral administration has been suggested as a stress-free, convenient, and cost-effective method for widespread vaccination and is preferred to the intramuscular way due to the lack of risk of secondary infections at the injection site (Naderi-Samani *et al.*, 2020).

In a recently safe and free of nano- or micro-encapsulation strategy performed in China, a recombinant live vector vaccine against IPNV was experimentally produced based on *L. Casei* and could induce protective immune responses in small fish via oral administration (Min *et al.*, 2012; Li-Li *et al.*, 2012). Lactic acid bacteria (LABs), generally known as harmless bacteria, are widely used in the food industry. In addition to their natural characteristics, such as lack of pathogenicity and the ability to improve food digestibility and reproduce in the gastrointestinal tract, these microorganisms are potentially capable of delivering antigens or DNA to mucosal surfaces. Cost-effective production and ease of use offer these bacteria as suitable vehicles for the production of oral vaccines (Naderi-Samani *et al.*, 2020).

In recent years, there have been efforts to use LABs for vaccinating fish against IPN (Min *et al.*, 2012; Duan *et al.*, 2018; Hua *et al.*, 2021). But to date,

no works have focused on cloning of a candidate gene of IPNV into *L. lactis* in order to express the protein product and deliver it via oral route to induce protective immune responses in fish. It seems there is an interesting novelty in this work in terms of oral vaccination of younger fish using a safe and strong genetically modified microorganism, *L. lactis*. With this purpose in mind, insertion of IPNV VP2 gene into pNZ8150 expression vector under the control of the NICE promoter and its cloning in *L. lactis* was investigated in the present study.

## Materials and methods

### *Viral isolate and bacterial strain*

In this study, DNA template for VP2 gene amplification was polymerase chain reaction (PCR) product of the gene (from ATG to stop codon, 1350 bp in length) from an Iranian isolate of IPNV (IRIPNV strain) as a generous gift from Dr. Maryam Dadar. Dadar *et al.* (2014) collected this isolate of IPNV from diseased fry of rainbow trout during an outbreak of IPNV from 2010 to 2012 in different fish farms in north, Mazandaran, and west, Chaharmahal and Bakhtiari and Kohgiluyeh and Boyer Ahmad, provinces of Iran.

Also, NZ9000 strain of *L. lactis* (MoBiTec GmbH, Germany) was used as both cloning and expression hosts. The *L. lactis* cells was cultured in 10 mL of SGLM17B medium containing 50% v/v M17 broth 2X (pH= 6.8, Sigma-Aldrich, United States) supplemented with 0.5% w/v glucose, 0.5% w/v lactose, 0.5 M sucrose, (and

1.5% agar for plate medium) and incubated overnight at 30°C (Mierau *et al.*, 2005). Then, the cells were stored long term via 15% glycerol.

## PCR

### *Primer design*

In pNZ8150 expression vector, nisA promoter is followed by *ScaI* site for translational fusions precisely at the start codon (ATG) (MoBiTec GmbH, 2015). Therefore, we intended to insert the IPNV VP2 gene between *ScaI* (at 5'-end of the forward primer) and *SpeI* (at 5'-end of the reverse primer) restriction sites from multiple cloning sites (MCS) of pNZ8150 expression vector. As the *ScaI* restriction enzyme produces a blunt end fragment, it didn't need to add the sequence of *ScaI* restriction site at the 5'-end of the forward primer. Nevertheless, *SpeI* creates a sticky end, so, we should consider the sequence of *SpeI* restriction site at the 5'-end of the reverse primer.

Because western blot analysis using anti-histidine tag (anti-histag) antibody is an experimentally common method to confirm recombinant protein expression in the host (Block *et al.*, 2009), a 6-histag sequence was considered at the 5'-end of the reverse primer (precisely before stop codon on the sense strand). Finally, the IPNV VP2 gene was amplified using specific primers designed by Oligo software (Offerman and Rychlik, 2003) which included 5'**ATGAACACAAACAAGGCAACC3**' as forward primer containing start codon (shown as bold letters) and 5'**AAAATTACTAGTTTAGTGATGGT**

*GATGGTGATGGACTATGTCTCTCC*  
*AGCACCA3'* as reverse primer including *SpeI* restriction site (shown as underlined letters), stop codon (shown as bold letters), and 6-histag (shown as italic letters).

#### *Gene amplification*

As DNA template for VP2 gene amplification was PCR product of the gene (from ATG to stop codon), four different PCR reactions were performed in a final volume of 50  $\mu$ L containing different PCR product concentrations (1, 5, 10, and 48 ng/ $\mu$ L), 1X reaction buffer, 200  $\mu$ M of each dNTP, 1 unit/reaction of *KOD* DNA polymerase, 1.5 mM  $MgSO_4$ , 0.3 mM of each forward and reverse primers, and double-distilled (dd)  $H_2O$  up to 50  $\mu$ L. PCR reactions were performed by a Gene cycler (Bio-Rad, Hercules, CA, USA) with the following thermo-cycling profile: initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for the 20s, 53°C for 10s, and 70°C for 27s. The final extension was 10 min at 72°C.

PCR products were visualized by electrophoresis on 2% agarose gel using 1X TAE buffer, stained with GelRed™ Nucleic Acid Gel Stain (Biotium, VWR, USA), and finally, imaging using ChemiDoc (Bio-Rad, USA).

In the next stage, PCR product was purified by high pure PCR template preparation kit (Roche Diagnostic, Germany) according to the manufacturer's protocol and the purified product was stored at -20°C until use.

#### *Gene cloning*

##### *Digestion*

Purified PCR product harboring *SpeI* restriction site was digested with *SpeI* restriction enzyme (Sambrook and Russell, 2001). Briefly, digestion reaction was carried out as follows: the reaction mixture containing 1X restriction buffer, 0.2  $\mu$ g of PCR product, 20 units of *SpeI* (Takara bio, Japan), and dd $H_2O$  up to a final volume of 100  $\mu$ L was incubated for 5h at 37°C. Then, 10 more units of *SpeI* were added to the mixture, and incubation was done again for 2h at 37 °C.

On the other hand, pNZ8150 expression vector was separately digested with *ScaI* (Takara bio, Japan) and *SpeI* restriction enzymes (as these two enzymes had different buffer conditions, it was not possible to double digest with the two enzymes simultaneously). Both digestion reactions were carried out with similar conditions as follows: the reaction mixtures containing 1X restriction buffer, 0.1  $\mu$ g of the vector, 10 units of enzyme (*ScaI* or *SpeI*), and dd $H_2O$  up to a final volume of 60  $\mu$ L were incubated for 2h at 37 °C. Then, 10 more units of enzyme (*ScaI* or *SpeI*) were added to the mixture, and incubation was performed again for 1h at 37°C.

##### *Ligation*

After dephosphorylation using alkaline phosphatase enzyme (Roche Diagnostic, Germany), ligation was performed with DNA ligation kit (Takara Bio Inc., Japan) as follows:

first, 0.03 pmol/reaction of linearized pNZ8150 vector (dissolved in TE, 10 mM Tris-HCl pH= 8.0, 1 mM EDTA) was mixed with 0.09 pmol/reaction of insert (PCR product dissolved in TE) in a total volume of 5  $\mu$ L. Second, an equal volume (5  $\mu$ L) of solution I was added to the DNA mixture. Finally, the combined solution was incubated at 16 °C for 30 min.

#### *Transformation*

Ligated mixture was transformed into *L. lactis* NZ9000 competent cells using electroporation. Electrocompetent cells of *L. lactis* NZ9000 were prepared according to the manufacturer's protocol (MoBiTec GmbH, Germany). First, 5 mL of G-SGLM17B medium containing 50% v/v M17 broth supplemented with 2.5% w/v glycine, 0.5% w/v glucose, 0.5% w/v lactose, 0.5 M sucrose was inoculated with *L. lactis* glycerol stock (from -80°C) and incubated in an anaerobic jar overnight at 30°C. Then, 50 mL of G-SGLM17B was inoculated with pre-culture in a dilution of 1:100 and grown overnight at 30°C, without aeration. 50 mL of full-grown culture was added to 400 mL of GL-SGM17B medium and grown until OD<sub>600</sub> was 0.2-0.3. Finally, the cells were washed four times by continual centrifuging at 6000g and 4°C for 20 min and resuspending in an ice-cold sterile solution containing 0.5 M sucrose and 10% glycerol. In the second washing, 50 mM EDTA was also added to the mentioned solution.

The recombinant expression plasmid (500 ng dissolved in TE) containing IPNV VP2 gene was transformed into competent *L. lactis* cells (40  $\mu$ L) by electroporation using a Genepulser system (BioRad, USA) and the parameters of 2000 V, 25  $\mu$ F, 200  $\Omega$ , and 5 ms pulse time in a pre-chilled 2 mm electroporation cuvette. Then, 1 mL of GLM17B medium supplemented with 20 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub> was added to the electroporated cells, and the cuvette was kept for 5 min on ice and incubated 1.5 h at 30°C. The transformed *L. lactis* cells containing the highest copy of the plasmid were screened by incubation on SGLM17 plates containing a high concentration (100  $\mu$ g/mL) of chloramphenicol (Sigma-Aldrich Chemie GmbH, Germany) for 2 days at 30°C.

#### *Colony PCR for recombinant plasmid verification*

The right colonies bearing the multi-copy of the "recombinant" vector, pNZVP2, grown on SGLM17 + chloramphenicol plates were initially verified and selected by colony PCR. All PCR conditions including materials and their concentrations and thermo-cycling profile were similar to the primary PCR as above mentioned, except for DNA template. Without any plasmid extraction from transformed colonies, DNA template was provided by boiling the cells at 96°C for 15 min (Sambrook and Russell, 2001).

### *Recombinant plasmid extraction and digestion*

The verified colonies using colony PCR were further examined by restriction enzyme digestion of extracted plasmid using *SpeI* as above mentioned. Digestion products were visualized by electrophoresis on 1% agarose gel using 1X TAE buffer, staining with GelRed™ Nucleic Acid Gel Stain, and imaging using ChemiDoc.

Before digestion reaction, the plasmids were extracted from transformed *L. lactis* colonies according to the manufacturer's protocol (MoBiTec GmbH, Germany), briefly as follows: first, 5 mL of SGLM17B medium was inoculated with the transformed *L. lactis* cells and incubated overnight at 30°C. The full-grown culture was spun down at 5000g for 10 min. Then, the pellet was resuspended in 250 µL THMS buffer (30 mM Tris-HCl pH=8, 3 mM MgCl<sub>2</sub>, 25% sucrose)+2 mg/mL lysozyme in a reaction tube and incubated for 10 min at 37°C. After that, 500 µL of 0.2 N NaOH+1% SDS was added to the mixture, shaken carefully (no vortex), and incubated for 5 min on ice. Then, 375 µL of ice-cold 3 M potassium acetate (pH 5.5) was added to the mixture, shaken carefully, incubated for 5 min on ice, and spun down for 5 min. In the next stage, the supernatant was taken out and added to a new reaction tube. The tube was filled with 2-propane and incubated for 10 min at room temperature. Finally, the mixture was spun down for 10 min and the pellet was washed using 70% ethanol,

vacuum dried, and dissolved in 50 µL TE.

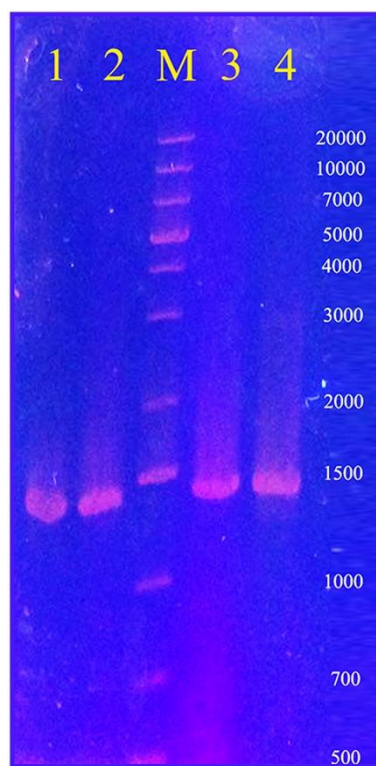
### *DNA sequencing for recombinant plasmid verification*

The verified colonies were, also, confirmed by DNA sequencing. Sequencing was performed for two samples in both directions using the gene specific primers as above mentioned, a BigDye terminator cycle sequencing reaction kit (Applied Biosystems, USA), and an ABI 3730 XL DNA Analyzer (Macrogen, South Korea). The achieved nucleotide sequence of the gene encoding IPNV VP2 and its deduced amino acid sequence were analyzed using BioEdit software (Hall, 1999).

## **Results**

### *PCR*

PCR product encoding VP2 gene of IPNV which contained *ScaI* restriction site and 6-histag sequence at the end of its sense strand was obtained using a primary PCR product as a template and a gene specific primer pair. Electrophoresis on agarose gel for the four different PCR reactions containing different primary PCR product concentrations (1, 5, 10, and 48 ng/µL) confirmed the all amplicons to be ~1.4 kb in length (Fig. 1).



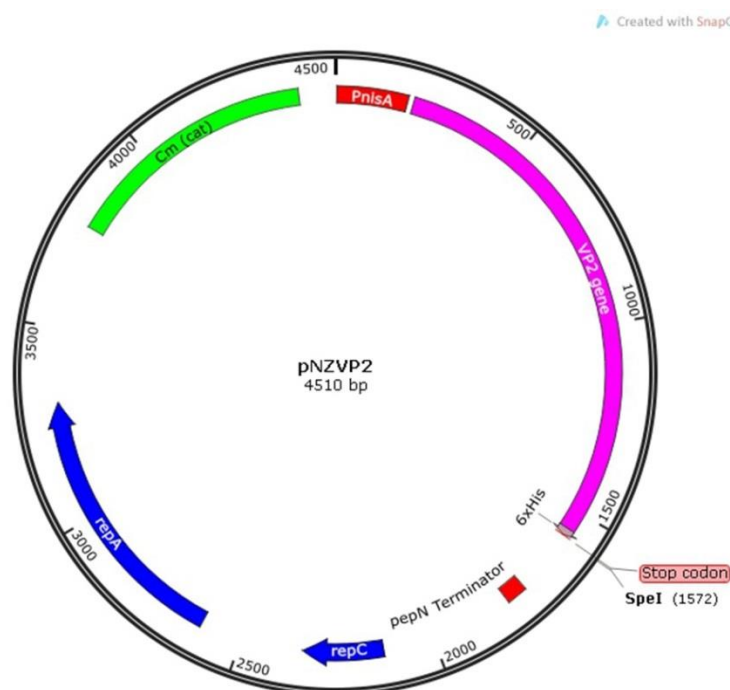
**Figure 1:** Electrophoresis on 2% (w/v) agarose gel for PCR products of IPNV VP2 gene amplified using four different primary PCR product concentrations (1, 5, 10, and 48 ng/μl), verifying the amplification of the gene with all four primary concentrations. Lanes 1, 2, 3, and 4: PCR products of IPNV VP2 gene amplified using 1, 5, 10, and 48 ng/μl of primary PCR product concentrations, respectively. Lane M: 1kb plus DNA ladder (Thermo Fisher Scientific, USA).

#### *Gene cloning*

PCR product was inserted into pNZ8150 expression vector between its *SpeI* and *ScaI* restriction sites (Fig 2). As shown in Figure 2, the recombinant IPNV VP2 gene is under the control of the *PnisA* (183 bp), a strong promoter from nisin A gene. Also, the 6-histag, stop codon and the *SpeI* restriction site are observed at the end of the gene. In pNZ8150, the termination sequence of *pepN* gene (52 bp) is used in order to stop transcription of the inserted gene. In order to screen the transformed colonies from non-transformed ones,

the chloramphenicol resistance gene, *Cm* (*Cat*), has been used as a selectable marker in pNZ8150. Although the recombinant construct, pNZVP2, seems to be 4537 bp in length (pNZ8150, 3163 bp; VP2 gene, 1347 bp; 6-histag, 18 bp; stop codon, 3 bp, and *SpeI* restriction site, 6 bp), it should be mentioned that a part of MCS region from pNZ8150 vector (27 bp) was removed by digestion using two restriction enzymes (*ScaI* and *SpeI*) since the final length of the recombinant construct was 4510 bp (Fig. 2).





**Figure 2:** Schematic representation of the recombinant expression vector, pNZVP2 (4510 bp), designed by SnapGene software version 3.3.4. The gene encoding VP2 of IPNV was cloned into the *ScaI/SpeI* restriction sites of pNZ8150 vector, in frame with *ScaI* site of pNZ8150. *PnisA*, the promoter from nisin A gene; *pepN* terminator, the termination sequence of *pepN* gene; *Cm (cat)*, the chloramphenicol resistance gene; *repA*, replication gene A; *repC*, replication gene C.

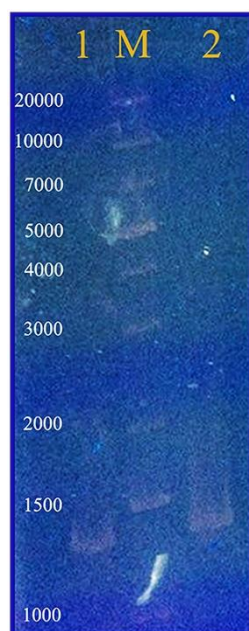
After ligation, the recombinant construct, pNZVP2, was transformed into *L. lactis* cells and multi-copy transformants were selected using high antibiotic concentration (10-fold increase of chloramphenicol concentration in screening plates than it in ordinary plates).

#### Colony PCR

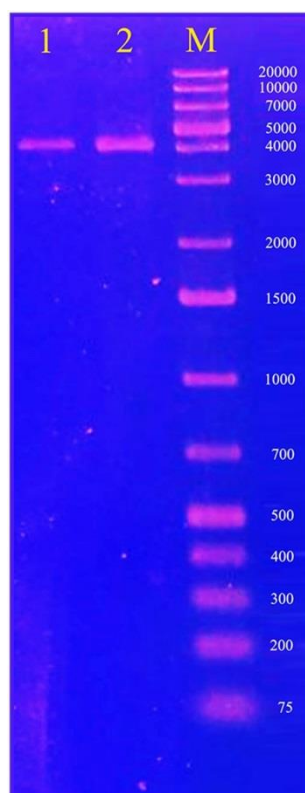
The recombinant construct, pNZVP2, was initially confirmed by PCR using DNA template obtained by boiling the cells from two different colonies grown on SGLM17 + chloramphenicol plates and a specific VP2 primer pair. Electrophoresis on agarose gel for both amplicons confirmed them to be ~ 1.4 kp in size (Fig. 3).

#### Restriction enzyme digestion

Construction of the recombinant expression vector, pNZVP2, was further verified using restriction enzyme digestion of the vectors extracted from two different colonies (initially verified using PCR) with *SpeI* restriction enzyme (Fig 4). As shown in Figure 4, electrophoresis on agarose gel confirmed the linearized recombinant vector (pNZVP2) from both colonies to have the higher length than the linearized non-recombinant one (pNZ8150), ~ 4.5 kb (Figs. 2 and 4) versus ~ 3.2 kb (Fig. 2) in terms of recombinant and non-recombinant vectors, respectively.



**Figure 3:** Electrophoresis on 2% (w/v) agarose gel for colony PCR products of IPNV VP2 gene amplified using two different transformed *L. lactis* colonies, verifying the construction of the recombinant expression vector, pNZVP2. Lane 1: 1kb plus DNA ladder (Thermo Fisher Scientific, USA). Lanes 2 and 3: PCR products of IPNV VP2 gene amplified using two different transformed *L. lactis* colonies.



**Figure 4:** Electrophoresis on 1% (w/v) agarose gel for restriction digestion of the vectors extracted from two different transformed *L. lactis* colonies (initially verified for construction of recombinant pNZVP2 expression vector using PCR) with *SpeI* enzyme, confirming the insertion of the gene encoding IPNV VP2 into pNZ8150 vector. Lanes 1 and 2: restriction enzyme digestion of the vectors extracted from two different transformed *L. lactis* colonies with *SpeI*. Lane M: 1kb plus DNA ladder (Thermo Fisher Scientific, USA).

### DNA sequence analysis

Formation of pNZVP2 expression vector was also proved by DNA sequencing. DNA sequence analysis confirmed the amplicon to be exactly 1374 bp in size including *SpeI* restriction site (6 bp), 6-histag (18 bp), stop codon (3 bp), and the coding sequence of IPNV VP2 gene (1347 bp) as reported from the sequence deposited in GenBank (accession number: KC489465) by Dadar *et al.* (2014).

Based on the sequence analysis between the IPNV VP2 gene sequence amplified and cloned in present work and the one released by Dadar *et al.* (2014), no mutations were found within the gene encoding VP2 from IPNV, confirming the high proofreading activity of *KOD* DNA polymerase.

### Discussion

There are many reports confirming antigenicity of the VP2 capsid protein of IPNV, since it has been identified as an immunodominant and non-infectious antigen (Tarrab *et al.*, 1995; Fridholm *et al.*, 2007; FROST *et al.*, 1998; Petit *et al.*, 2000). In this regard, most of the research teams have used this gene to produce a recombinant vaccine against IPNV (Allnutt *et al.*, 2007; Dhar *et al.*, 2010; Ana *et al.*, 2009; Ballesteros *et al.*, 2015; Ahmadvand *et al.*, 2017; Dadar *et al.*, 2014).

Although some recombinant IPNV SVPs including VP2 protein expressed by yeast (Allnutt *et al.*, 2007; Dhar *et al.*, 2010) and also recombinant VLPs produced by mammalian and fish cell lines (Labus *et al.*, 2001) and

baculovirus insect expression system (Shivappa *et al.*, 2004) have given a significant protection like several oral recombinant vaccines (Min *et al.*, 2012; Embregts and Forlenza, 2016; Ahmadvand *et al.*, 2017), these protections were obtained by non-oral routes such as intraperitoneally injection. Because the initial infection and pathogen replication of IPNV is mainly occurred at the mucosal surfaces (Min *et al.*, 2012) and in fingerling stages (Cuesta *et al.*, 2010; Wolf *et al.*, 1960; Biering *et al.*, 2005; Roberts and Pearson, 2005), these vaccine delivery strategies are not applicable for younger fish, and oral vaccination route is preferred.

In terms of commercial recombinant IPNV vaccines, it should be mentioned that the one developed based on recombinant VP2, Norvax® Protect-IPN (NP-IPN), and licensed in Norway (Frost and Ness, 1997), Canada, and Chile (Woo and Cipriano, 2017) is an injectable vaccine which has shown different protection levels in field studies (Ramstad and Midtlyng, 2008, Ramstad *et al.*, 2007). The other commercial IPNV vaccine licensed in Chile, AquaVac\* IPN Oral (Merck Animal Health, New Jersey, USA), is an oral vaccine based on VP2 and VP3 recombinant antigens produced by yeast expression system (Woo and Cipriano, 2017; Cuesta *et al.*, 2010). However, this disease is still widespread in Chile (Manríquez *et al.*, 2017; Tapia *et al.*, 2015) since in 2015, about 30% of disease diagnoses reported from Chilean salmon farming centers were

related to IPNV which caused about 8% of pathogen associated mortalities (Manríquez *et al.*, 2017). Even if the significant protection of this commercial vaccine is confirmed (Cuesta *et al.*, 2010), production of the vaccine is complicated and is not cost effective because of some additional and high cost processes including cell lysis, antigen purification, and nano-encapsulation.

Another strategy for immunizing the small fish against IPNV is oral DNA vaccination (Ahmadivand *et al.*, 2017; Evensen and Leong, 2013; Gudding *et al.*, 2014; Hølvold *et al.*, 2014; Embregts and Forlenza, 2016). The first DNA vaccine has been licensed in 2005 for the infectious hematopoietic necrosis virus (IHNV) as a rhabdovirus in Canada (Appex-IHN, Aqua Health Ltd.). Nevertheless, DNA vaccines for non-rhabdoviruses confer lower protection in fish (Lorenzen and LaPatra, 2005, Kurath, 2008, Tonheim *et al.*, 2008). Although some of the DNA vaccine plasmids encoding VP2 protein showed a high protection against IPNV (Embregts and Forlenza, 2016; Ahmadivand *et al.*, 2017; Cuesta *et al.*, 2010), DNA vaccines are needed to be encapsulated via nano- or micro-particles to be protected in gastrointestinal tract (Ahmadivand *et al.*, 2017; Ballesteros *et al.*, 2015; Embregts and Forlenza, 2016). Furthermore, these vaccines always encounter some ethical issues in terms of biosafety. As a matter of fact, a DNA vaccine plasmid is possible to integrate into the host cell genome (Temin,

1997), causing insertional mutagenesis which can potentially activate oncogenes or inactivate tumor suppressor genes (Wolff *et al.*, 1992; Smith, 1994).

Therefore, an ideal vaccine against IPNV must be a “safe” and “cost effective” one inducing “long lasting protection” at an “early age” using oral delivery route (Min *et al.*, 2012). In this regard, lactic acid bacteria (LAB) as live vector vaccines have these abilities. Before using as cell factories for industrial production of recombinant vaccines (Lam and Xu, 2013; Pouwels *et al.*, 1996; Cano-Garrido *et al.*, 2015; Claassen *et al.*, 1995), LAB have been extensively utilized for centuries as the useful microorganisms (probiotics) for fermentation and preservation of a large number of industrial dairy and other food products (Liu *et al.*, 2005; Gareau *et al.*, 2010; Konings *et al.*, 2000; Ross *et al.*, 2002). They have been classified as food grade microorganisms [generally recognized as safe (GRAS) organisms by the US Food and Drug Administration (FDA)] and fulfil criteria of the qualified presumption of safety (QPS) according to the European Food Safety Authority (EFSA) (Cano-Garrido *et al.*, 2015). Most strains of LAB are able to survive and colonize on the intestinal tract without any side effects for the host (Galdeano and Perdigon, 2006; Ogawa *et al.*, 2006; Li-Li *et al.*, 2012).

To date, LAB have been orally administered to deliver exogenous antigen on the mucosal surface to induce local mucosal and systemic

immune responses in mice (Liu *et al.*, 2011; Villena *et al.*, 2011), swine (Hou *et al.*, 2007), and fish (Li-Li *et al.*, 2012; Min *et al.*, 2012). Min *et al.* (2012) and Li-Li *et al.* (2012) selected *L. casei* as a live vector expressing and delivering IPNV VP2/VP3 capsid protein in the separate and fusion forms, respectively, via oral route to induce protective immune responses in younger fish. They reported that both separate and fusion forms of IPNV VP2/VP3 proteins were successfully expressed in *Lactobacillus* with natural antigenicity and were capable of inducing antibodies against natural IPNV and significant reductions in viral loads. However, these vaccines have not been licensed for commercial production yet.

The other important member of the LAB is *L. lactis* which is a homofermentative bacterium and is best known for its use in the manufacture of cheese and other dairy products (Platteeuw *et al.*, 1996). At present, the full genome information of several strains of *L. lactis* is publicly available (<https://www.ncbi.nlm.nih.gov/nucleotide/?term=lactococcus+lactis+complete+genome>). This wealth of knowledge and experience has led to the use of lactococci in several fields of biotechnology, specially expression of bacterial and viral antigens for safe vaccination via mucosal immunization (Bermúdez-Humarán *et al.*, 2013; Wells, 2011; Villatoro-Hernández *et al.*, 2012; Dolatabadi *et al.*, 2015). The availability of an easy-to-operate and strictly controlled gene expression

system, NICE (Nisin Controlled Expression), has been crucial for the development of many of these applications. By using this system, high expression levels of the proteins are achieved using a food-grade molecule (nisin) as an inducer for PnisA promoter (Mierau and Kleerebezem, 2005; De Ruyter *et al.*, 1996; Kleerebezem *et al.*, 1997; Mierau *et al.*, 2005).

Recently, a US patent (publication number: US 20130004547 A1) was published by Lam and Xu (2013) as the inventors of the oral vaccines produced by *L. lactis* strains. In one embodiment, *L. lactis* expressing the avian influenza HA gene could be used as an oral vaccine for protection against H5N1 virus infection. In another embodiment, *L. lactis* was administered as an oral vaccine in conjunction with an adjuvant such as cholera toxin B.

In the present study, the VP2 gene from an Iranian isolate of the IPNV was successfully inserted between *ScaI* and *SpeI* restriction sites of the pNZ8150 expression vector under the control of PnisA promoter. Then, the recombinant expression vector, pNZVP2, was transformed and cloned into *L. lactis* NZ9000. Construction of the recombinant expression vector, pNZVP2, was verified using restriction enzyme digestion, colony PCR of the VP2 gene and DNA sequencing. We, also, added a 6-histag sequence at 3' end of the sense strand corresponding to C-terminal of the VP2 protein to verify protein expression in the host cell, *L. lactis* NZ9000, using anti-histag in

western blot analysis for our future expression studies.

Similarly, Man-Kit Lam and Xu (2013) used pNZ8150 expression vector in order to transform the HA gene of Influenza virus H5N1 into *L. lactis*. In contrast with our work, they constructed the recombinant expression vector, pNZ8150-HA, through the insertion of the HA gene at the “*SpeI/HindIII*” restriction sites of the pNZ8150 expression vector.

Present study could be the first stage of producing an oral live vector vaccine against IPNV. Indeed, other complimentary stages including expression and immunization studies on live fish were remained to investigate in the future.

The accuracy of the gene sequence amplified using DNA polymerases in PCR is very important for recombinant protein expression. Some of the DNA polymerases such as *Taq* lack the 3'-5' exonuclease activity while the others such as *Pfu* and *KOD* are able to do proofreading activity (Null and Muddiman, 2001; Takagi *et al.*, 1997). Moreover, processivity, the number of nucleotides extended before the polymerase detaches, is 5–20 times higher for *KOD* compared to *Taq* or *Pfu* (Null and Muddiman, 2001; Takagi *et al.*, 1997). This property along with the exonuclease activity of *KOD* decreases the chance of mis-incorporation of nucleotides during the PCR (Benson *et al.*, 2003). Furthermore, the higher extension rate (2- to 4-fold) of *KOD* compared to *Taq* or *Pfu* results in a shorter amplification time to generate

an equivalent amount of amplicon (Benson *et al.*, 2003). Therefore, in the present study, this enzyme was used for amplification of VP2 gene. As, in present work, DNA template for PCR was the primary VP2 PCR product previously amplified by Dadar *et al.* (2014), it was expected to observe no differences between these two sequences (except for restriction sites and 6-histag). Comparison of the DNA sequence between the VP2 gene amplified in present work and the one deposited in GenBank (accession number: KC489465) by Dadar *et al.* (2014) showed no mutations within the sequence which verified the high proofreading activity of *KOD*.

To the best of our knowledge, this is the first study applying *L. lactis* to clone a candidate gene from a pathogen infecting fish regarding the production of a recombinant live vector vaccine. As IPNV has resulted in high losses in fish in Iran and all over the world, we focused on an antigenic protein, VP2, of this virus as a model. However, it seems this expression system can be used for expression and oral delivery of not only IPNV antigens, but also, for any other infectious agents via pNZ8150/*L. lactis* expression system, potentially as a novel platform for the development of oral delivery vaccines in fish.

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